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PRINCIPAL INVESTIGATOR: Timothy G. Whitsett, Jr.
Coral A. Lamartiniere, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham AL 35294-0109

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14. ABSTRACT: Breast cancer a destructive disease despite new therapeutics. It is well accepted that environmental factors, especially diet, can play an important role in determining one's future risk of the disease. We believe that two natural polyphenols, genistein (a component of soy) and sesveratrol (a component of grapes and red wine), can suppress mammary carcinogenesis. We and others have clearly shown a mammary-protective effect against chemically-induced mammary cancer. This pronet aims to elucidate mechanisms through which these polyphenols may exert their effects. We show that genistein and resveratrol can modulate the protein expression of several critical proteins in the mammary gland that are involved in growth and proliferation. We see changes in both the MAPK signaling pathway, the PI3K/Akt pathway, as well as changes in sex steroid receptor co-activators. We have demonstrated that the estrogen receptors play an important role in the mechanisms of genistein and resveratrol. Lastly, we show that a novel mouse model that over-expresses AIB1 may not be suitable for early exposure, chemoprevention experiments.					
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Introduction:

Breast cancer remains a destructive disease and a leading killer among women in the United States and throughout the world [1]. It has been recognized that genetic alterations (such as BRCA 1 and 2 mutations) account for only 10-15% of breast cancer. Thus, environmental exposures, especially diet, can play a very important role in the causation or prevention of this disease. We believe that the dietary polyphenols genistein, the major phytoestrogen in soy, and resveratrol, a phytoalexin found in red grapes and red wine, can protect a woman against mammary carcinogenesis. We, and others, have shown that dietary exposure to genistein or soy, especially early in life, can protect against chemically-induced carcinogenesis [2-3]. We demonstrated that prepubertal exposure to genistein caused a significant reduction in terminal end buds, the most susceptible structures for mammary carcinogenesis. We and others have also shown a protection against breast cancer in a chemically-induced rat model with exposure in the diet to resveratrol [4-6]. Resveratrol caused a significant reduction in mammary tumor multiplicity and increased tumor latency. The epithelial cells of the terminal end buds show a significant reduction in proliferation and increase in apoptosis, which might help to explain the chemoprotection that we observed [6]. With observations from the tumorigenesis studies, mammary gland maturation, and cell proliferation experiments; we proposed to look for changes at the molecular level that could account for the protection we observe with dietary genistein and resveratrol. We propose to focus on sex steroid and growth factor signaling pathways, and the steroid receptor coactivator family, a possible link between critical sex steroid and growth factor pathways. Also, we want to better understand the role of the estrogen receptors in the chemopreventive mechanisms of genistein and resveratrol. Understanding the *in vivo* mechanisms of these polyphenols will allow them to be used to protect women against breast cancer.

Body:

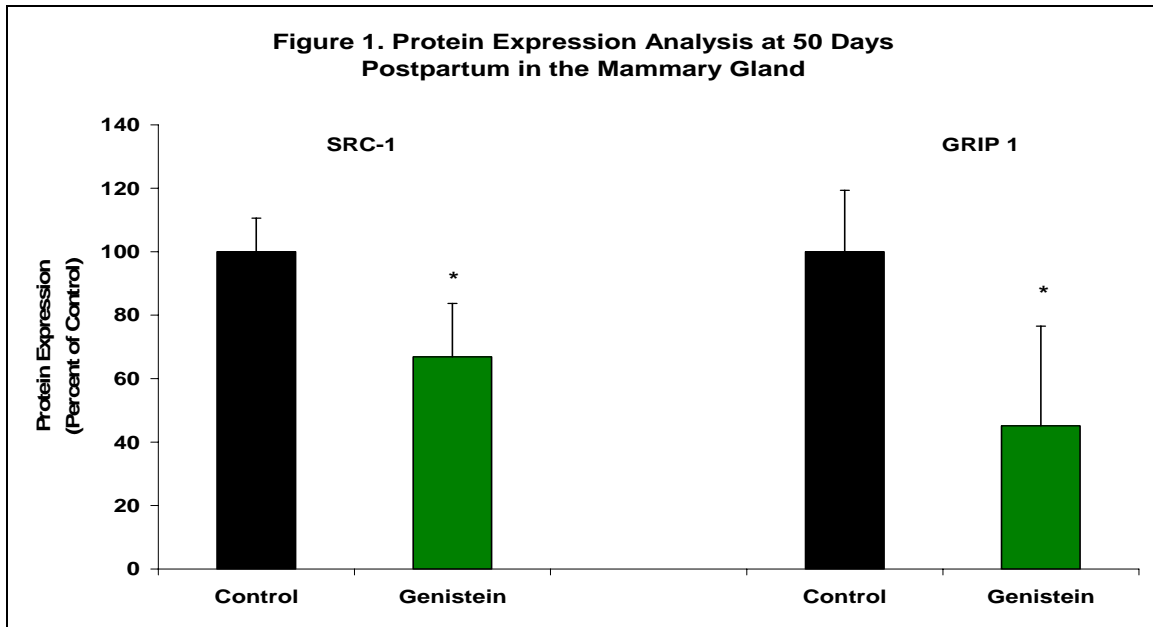
To discuss the research accomplishments in the first two years (Feb. 2005 – Feb. 2007), the original Statement of Work will be used with each of three aims being discussed.

Aim 1. (Months 1-12). As a means of understanding how genistein and resveratrol suppress the development of chemically-induced mammary cancer in rats, we proposed to investigate the potential of genistein and resveratrol, alone and in combination, to regulate critical sex steroid receptors, steroid receptor coactivators, and critical growth factors and receptors in the mammary glands of Sprague- Dawley rats.

The fourth abdominal mammary glands were dissected from both 21- and 50-day-old rats treated \pm the polyphenols genistein and resveratrol, alone and in combination.

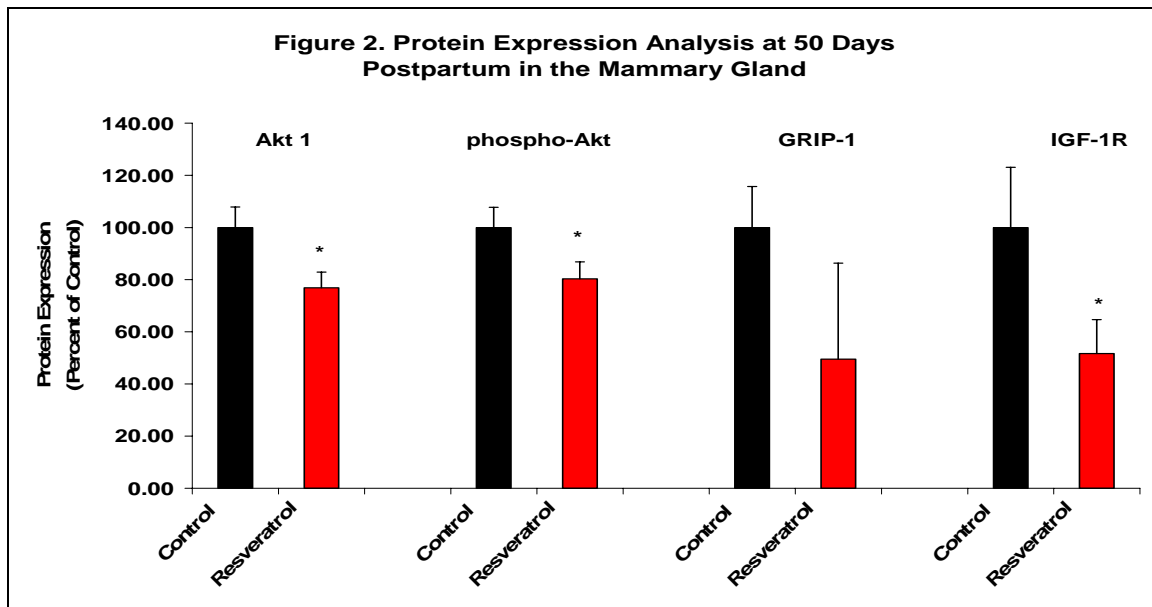
Immunoblot analysis was employed to look at the protein expression of several sex steroid receptors, steroid receptor coactivators, and growth factor receptors. The steroid receptor coactivator GRIP-1 (GR interacting protein-1) was shown to be up-regulated at 21 days postpartum by genistein in the diet (data not shown). This is followed by a decrease in GRIP-1 expression at 50 days postpartum (Figure 1). This fits a model proposed by our lab that genistein causes an increase in mammary gland proliferation and maturation early in the animals, followed later in life by a more mature gland that is less proliferative and thus less susceptible to carcinogenesis [reviewed in 7]. More recently,

with genistein treatment, we observed a decrease in the expression of SRC-1 (steroid receptor coactivator-1), another coactivator that enhances steroid receptor signaling, at 50 days postpartum. We did not observe significant differences in the sex steroid receptors such as the estrogen receptors alpha and beta or progesterone receptors at 21 or 50 days postpartum. We did not observe significant regulation of the epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF-1R), or total and phosphorylated extra-cellular regulating kinase 1&2 (ERK 1&2) at 21 or 50 days postpartum (data not shown).



*Figure 1. Rats treated \pm genistein (250mg/kg diet) were sacrificed at 50 days postpartum. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Control set to 100. * represents a p value < 0.05*

Resveratrol-treatment resulted in protein modulation which helps to explain the protection that we observed against mammary carcinogenesis. At 50 days postpartum, we observed a significant reduction in the level of GRIP-1 (Figure 2), similar to what we observed with genistein treatment. Furthermore, we detected decreases in protein expression of IGF-1R, Akt, and the phosphorylated (active) form of Akt. All of these have been implicated in mammary cell proliferation and carcinogenesis. A reduction in these growth factors may help to explain the mammary chemoprevention that we observed in the rat model. Again, we observed no difference in the protein expression of the estrogen receptors or the progesterone receptors at 21 or 50 days postpartum. We saw no changes in the steroid coactivators at 21 days postpartum. At 50 days postpartum, there was a trend toward reduction in the phosphorylated forms of ERK 1 and 2, but it did not reach statistical significance (data not shown). Thus, resveratrol and genistein may exert chemoprotective actions on growth factor signaling molecules, and the coactivator molecules for the sex steroid receptors.



*Figure 2. Rats treated \pm resveratrol (1000mg/kg diet) were sacrificed at 50 days postpartum. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Control set to 100. * represents a p value < 0.05*

The combination of genistein and resveratrol treatments did not reveal any changes that were not observed in either of the single administrations (data not shown). This fits in with our recent data that showed combinations of genistein and resveratrol were protective against mammary carcinogenesis, but not any better than either agent by itself [8]. Treatment with estradiol in the diet is currently under investigation at 50 days postpartum and will serve as an estrogenic control using dietary administration.

Aim 2. (Months 12-36). The goal of this aim was to investigate if genistein and resveratrol act independently of the estrogen receptors. This was carried out in bilaterally ovariectomized rats to eliminate endogenous ovarian estrogens and the use of ICI 182,780 to block estrogen receptors- α and β (ER- α and β). Estradiol benzoate (EB) was used as a positive, estrogenic control.

The fourth abdominal mammary glands and the uteri were dissected from rats that had been ovariectomized at 7 weeks of age and injected with a subcutaneous (s.c.) dose of genistein (500 mg/kg BW), resveratrol (500 mg/kg BW), estradiol benzoate (500 ug/kg BW), or vehicle control (equivalent volume of DMSO) after three weeks of recovery (10 weeks of age). The subcutaneous dose followed pre-treatment \pm the pure antiestrogen, ICI 182,780 (4 mg/kg BW) to block the estrogen receptors. A look at the uterine to body weight ratios (Table 1) demonstrates the effectiveness of the ICI 182,780 treatments. This is especially evident with the estradiol benzoate injection with or without ICI 182,780 pretreatment. Without ICI 182,780 pretreatment, EB resulted in a statistically significant

increase (74% compared to the control group) in the uterine to body weight ratio, suggesting uterine growth and proliferation as would be expected with an estrogenic agonist. Blocking the estrogen receptors by ICI 182,780, the uterine to body weight ratio was not significantly different from the control group following EB injection. We would have expected the ICI + EB group to result in a greater reduction in uterine to body weight ratio (more similar to the control group), and thus may increase the dose of ICI 182,780 in future experiments. It should be noted that genistein and resveratrol had no statistically significant effect on uterine to body weight ratios, although genistein-treated rats displayed a 34% increase compared to the control group. This would fit with the characterization of genistein as a weak estrogen agonist. As one would expect, the uterine to body weight ratio of the Sham group (ovary intact) was significantly increased compared to any rat that was ovariectomized.

Treatment	Uterine to Body Weight Ratio	Percent of Control
Sham (ovary intact)	2.07 ± 0.23 *	581
Control (DMSO)	0.36 ± 0.02	100
Estradiol Benzoate (EB)	0.62 ± 0.09 *	174
Genistein	0.48 ± 0.11	134
Resveratrol	0.39 ± 0.04	110
ICI + Control	0.34 ± 0.02	96
ICI + EB	0.48 ± 0.02	134
ICI + Genistein	0.38 ± 0.03	106
ICI + Resveratrol	0.31 ± 0.03	87

Table 1. Ovariectomized rats were injected subcutaneously with polyphenols or estrogen with or without pretreatment with ICI 182,780. Uteri were dissected and weighed to obtain uterine to body weight ratios.

** represents a p value < 0.05 as compared to the Control group.*

As mentioned above, the fourth abdominal mammary gland was removed from rats that had been ovariectomized to remove endogenous estrogens, pretreated ± the antiestrogen ICI 182,780, and then treated with a single, subcutaneous dose of estradiol benzoate, genistein, or resveratrol. I will discuss the protein expression results for each estrogen or phytoestrogen individually.

Estradiol benzoate. As seen in Table 1, a single s.c. dose of estradiol benzoate was able to significantly increase the uterine to body weight ratio compared to Control (DMSO) group. We expected to see a significant decrease in protein levels of ER α and β along with an increase in progesterone receptor (PR) levels. We and others have shown that activation of the estrogen receptors by estradiol causes a reduction in receptor levels hypothesized to be associated with receptor ubiquitin-proteosomal degradation [9]. An increase in mRNA and protein levels of PR is one of the classical responses to ER activation due to an estrogen responsive element in the progesterone receptor gene promoter region. We also expected all of these responses to be blocked by pretreatment with ICI 182,780. Surprisingly, we observed no changes in the estrogen receptor protein levels (although the trend for ER β was down-regulation). We also observed no change in

PR protein expression levels. One explanation for these observations is that these actions (decreased ER and increased PR) may have already occurred by our sacrifice time of 16 hours after estrogen exposure. Another possibility is that the activity levels of the receptors are modulated with no change at this time point in receptor protein levels. We are currently running these blots again to ensure that our observations were accurate. We observed no significant change in the steroid receptor coactivators (SRC-1, GRIP-1, or AIB1). Treatment with EB tended to increase the levels of EGFR, an event that was not abrogated by pre-treating the rats with ICI 182,780. This confirms the possibility that estrogens can affect growth factor pathways through non-estrogen receptor pathways. Interestingly, we observed increases in both the phosphorylated (active) forms of Akt and ERKs1&2 (Figure 3), although the results did not reach statistical significance. These increases may help to explain the estrogenic growth effects that were observed in the uterus. The actions on phospho-Akt and phospho-ERKs 1&2 were abrogated with pretreatment of ICI 182,780, suggesting a mechanism that involves the estrogen receptors.

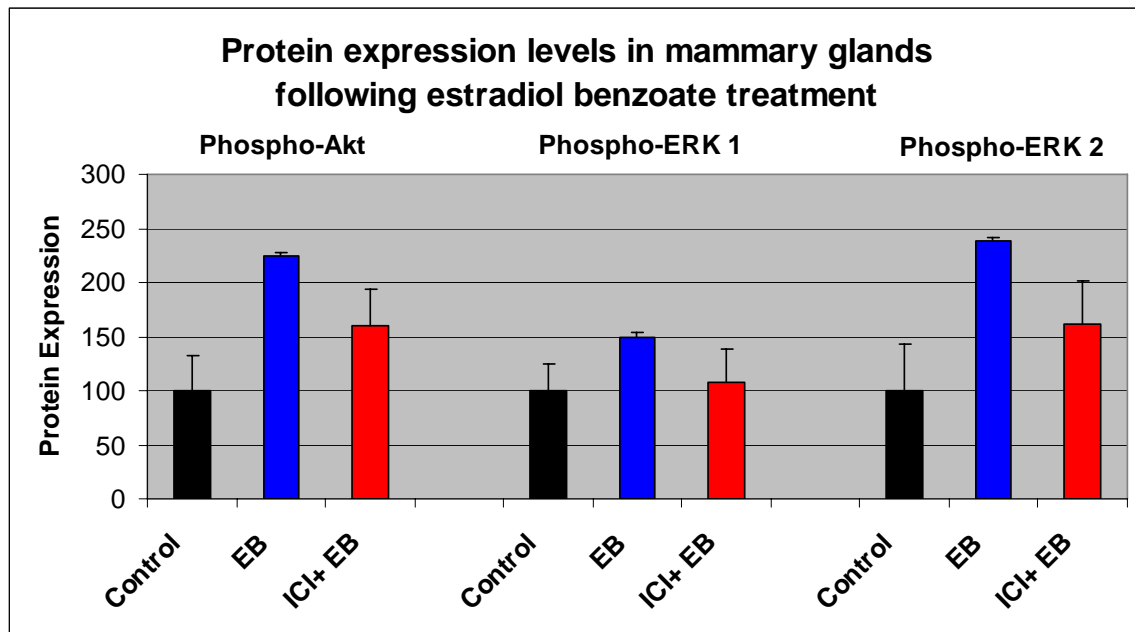


Figure 3. Ovariectomized rats treated \pm ICI followed by EB were sacrificed. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Controls set to 100.

Genistein. The effect of removing endogenous estrogens, using s.c injection as the route of administration, and a pharmacologic dose of genistein (500 mg/kg BW) seemed to have the greatest effect on the actions of genistein in the rat mammary gland, compared to those effects seen after dietary exposure. The significant decreases in SRC-1 and GRIP-1 that we observed following dietary administration (Figure 1) were not present in this ovariectomized model following genistein injection. All three steroid receptor coactivators tested (SRC-1, GRIP-1, and AIB1) tended to have increased protein expression compared to the control group, although none of the three reached statistical significance (data not shown). Interestingly, pretreatment with the antiestrogen ICI

182,780 resulted in levels more similar to the control group, implicating that genistein may have actions through the estrogen receptors. The affinity of genistein for ER-beta has previously been reported [10]. To this point, we expected a reduction in the protein levels of ER-alpha and beta as has been seen previously [9]. Protein levels of ER- alpha and beta were unchanged compared to the control group. As with estradiol benzoate treatment, we may have missed this action at the 16 hour time point that was used in this study. We also observed no significant differences in the protein levels of IGF-1R, the phosphorylated forms of Akt and ERKs 1 & 2, PR, or total ERK1s & 2 in this model, similar to what we reported after the administration of genistein in the diet (Aim1).

Resveratrol. In this model (ovariectomy and s.c. injections), resveratrol caused protein expression changes that may help to elucidate the mammary chemoprotective effects that we and others have observed [4-6 and 8]. Exposure to resveratrol resulted in a statistically significant reduction in both ER α and PR b (Figure 4). Pretreatment with ICI 182,780 abrogated both of these protein modulations. Both the estrogen and progesterone receptors are known to play a role in mammary gland growth, proliferation, and disease progression and a decrease may help to explain the protective mechanisms of resveratrol. SRC-1 was also down-regulated by resveratrol treatment, though it did not reach statistical significance (data not shown). This effect was not blocked by ICI 182,780 pretreatment suggesting a non-ER mediated effect. A decrease in this coactivator might also help to explain the protective effects of resveratrol as the coactivators allow for more efficient transcriptional activity of the steroid receptors such as ER and PR. We observed no change in the levels of co-activators AIB1 and GRIP-1 in this model (data not shown).

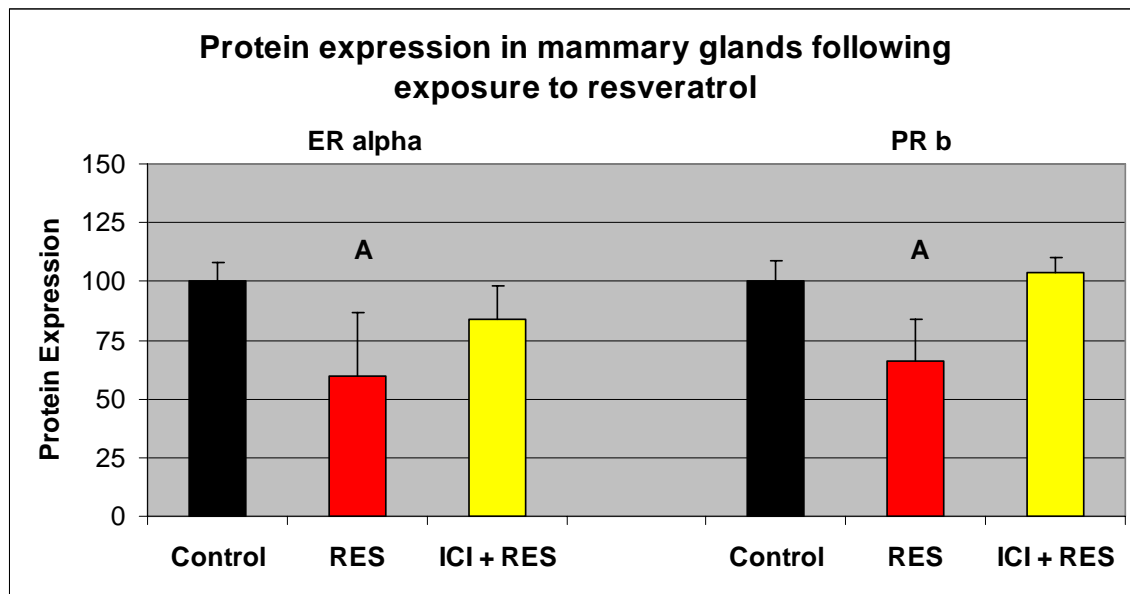


Figure 4. Ovariectomized rats treated \pm ICI followed by resveratrol treatment were sacrificed. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Controls set to 100. A represents a p value < 0.05

Not just steroid receptors were affected by treatment with resveratrol. We also observed changes in growth factors that are known to play a role in mammary development and disease. EGFR protein expression was significantly decreased following resveratrol injection (Figure 5). This action was inhibited by pretreatment with the antiestrogen, ICI 182,780. A statistically significant decrease in the phosphorylated (active) forms of Akt and ERKs 1&2 was also observed (Figure 5). It should be noted that this is the opposite effect observed following EB treatment (increased phospho-Akt and ERKs 1&2 protein levels). This effect was also seen in Aim 1 (Figure 2) with the administration of resveratrol in the diet. Interestingly, pretreatment with a pure antiestrogen (ICI) resulted in no change in the protein levels of phospho-Akt and ERKs 1&2. This suggests a role for the estrogen receptors in the mechanisms observed for resveratrol. Cross-talk between the estrogen receptor and several growth factor pathways (EGF and IGF pathways) have been suggested *in vitro* and certainly warrant further *in vivo* investigation in the chemopreventive mechanisms of resveratrol. We observed no differences in the protein expression of total ERK 1&2 (data not shown).



Figure 5. Ovariectomized rats treated \pm ICI followed by resveratrol treatment were sacrificed. Mammary glands were dissected and assayed for protein expression by western blot analysis. Results are given as means + SEM, with the Controls set to 100. A represents a p value < 0.05

There are still proteins that are under investigation (Akt, PR a, and others) in each of the treatments used in this ovariectomized model. There are also proteins that will be re-run to ensure for accuracy of the data.

Aim 3. (Months 1-36). The goal of this aim was to investigate the potentials of genistein and resveratrol to suppress mammary tumorigenesis in a novel mouse model that over-expresses AIB1 [11]. The over-expression of human AIB1 in these mice is sufficient to cause tumorigenesis, although there is a long latency period (up to two years).

Our colony of AIB1 transgenic mice expanded such that we had enough mice to run the mammary cancer chemoprevention experiment as well as a detailed ontogeny study. In the ontogeny study, we looked at four mice every month to get a better understanding of the onset of lesions and tumors in this novel model. We had enough mice to sacrifice and evaluate at least four mice every month from months 4-24 postpartum. As the ontogeny study progressed, we discovered an alarming trend. From months 4 to 18, we no tumors were detected via palpations and no pathologic lesions were found by a UAB board certified pathologist. We sent DNA samples from all of the colony founders and several offspring to ensure that the mice were transgenic. The lab of Dr. Myles Brown (principle investigator of the first characterization of this model) confirmed that all of the mice were transgenic by PCR analysis. The original characterization of the model observed lesions as early as 5 months of age [11]. Another problem arose as our aged mice started to die of problems not related to mammary tumorigenesis (especially stomach and rectal pathologies). With the length of time that the tumors take to develop (> 2 years) and the absence of early, characterized lesions, we are forced to take the stance that this is not an attractive model for mammary cancer chemoprevention using these polyphenols.

Another factor played into our decision to end the chemoprevention experiment with this particular model. This model is based on an over-expression of the steroid coactivator AIB1. We have not been able to confirm that AIB1 can be significantly regulated by the polyphenols genistein and resveratrol, alone or in combination. We strongly believe that both genistein and resveratrol can protect the mammary gland against carcinogenesis, but at this time do not think that the protein modulation of AIB1 is a major mechanism. This makes this mouse model the wrong choice for chemoprevention experiments with these polyphenols.

Key Research Accomplishments:

- Significant modulation of several proteins by genistein and resveratrol was detected in Aim 1. Many of these are important for mammary growth, proliferation, and chemoprevention by genistein and resveratrol at 50 days postpartum.
- The use of ovariectomy and blocking of the estrogen receptors were effective as evidence by uterine to body weight ratio changes. In this model, estradiol benzoate was able to modulate growth factor pathways. Resveratrol was able to affect both sex steroid pathways and growth factor paths that are involved in mammary gland development and carcinogenesis.
- Based on data from Aims 1 and 2, we believe that the timing of exposure, the route of administration, and the dose of genistein treatment can significantly impact the mechanism of action in the mammary gland. The protective effects that were observed after dietary administration (decreased levels of SRC-1 and GRIP-1) were not observed after pharmacologic injection in ovariectomized rats.

- A manuscript to the Journal of Carcinogenesis (2006) was accepted dealing with the chemopreventive properties of resveratrol. This work has become one of the ten most viewed papers of the past year in the journal.
- Data and ideas stemming from this grant were used to publish a review article in Expert Reviews in Anticancer Therapies (2006).
- Data from this project was used to attend a conference and present a poster at the 2005 Gordon Research Conference: Hormonal Carcinogenesis. A graduate student fellow award was received for the work.
- Data from this project was used to attend and present a poster at the 2005 Society of Toxicology national meeting. A graduate student travel award was received for the work.
- Data from this project was used to attend and present a poster at the 2005 and the 2006 UAB Comprehensive Cancer Center Annual Research Retreat. The PI (Tim Whitsett) received the prestigious William Bailey Award for Excellence in Cancer Prevention and Control for this work.
- Data from this project was accepted for poster presentation for two separate meetings in 2006: AACR annual meeting and the Gordon Research Conference: Mammary Gland Biology.
- Some of the data obtained from this project was used to help the PI (Tim Whitsett) qualify into candidacy in the UAB Pharm/Tox doctoral program.

Reportable Outcomes:

PUBLICATIONS:

Whitsett T, Lamartiniere CA. Genistein and resveratrol: mammary cancer chemoprevention and mechanisms of action in the rat. *Expert Rev. Anticancer Ther.*, 6(12), 2006.

Whitsett T, Carpenter DM, Lamartiniere CA. Resveratrol, but not EGCG, in the diet suppresses DMBA-induced mammary cancer in rats. *J. Carcinog.* 5:15, 2006.

Some of the results from this grant have been used in poster presentations at national scientific meetings over the past two years.

ABSTRACTS:

Whitsett T, Chen D, Lamartiniere C. Mammary cancer chemoprevention with the polyphenols genistein and resveratrol. Third Annual Early Environmental Exposures Meeting, Breast Cancer and Environment Research Centers. Nov. 2006.

Whitsett T, Chen D, Lamartiniere C. Mammary cancer chemoprevention with the polyphenol resveratrol. Gordon Research Conference: Mammary Gland Biology. June 2006.

Whitsett T, Chen D, Lamartiniere C. Mammary cancer chemoprevention with the polyphenol resveratrol. AACR Annual Meeting. Proceedings, Volume 47, p. 534. April 2006.

Whitsett T and Lamartiniere CA. Breast Cancer Chemoprevention with the Polyphenol Resveratrol. Gordon Research Conference: Hormone Action in Development and Cancer. July 2005.

A graduate student fellow award was received for the work.

Whitsett T and Lamartiniere CA. Breast cancer chemoprevention with the polyphenol resveratrol. Society of Toxicology 44 Annual Meeting. Program page 164. March 2005. A student travel award was received for the work.

The data was also used in an invited seminar at UAB. The title of the seminar was: "Mammary Cancer Chemoprevention with the Polyphenols Genistein and Resveratrol" UAB Pharmacology and Toxicology Seminar, Feb. 2006

Conclusions:

We and others have clearly shown a protection against mammary carcinogenesis using the polyphenols genistein and resveratrol in the diet. We have also shown the ability of these polyphenols to modulate mammary gland maturation, as well as cell proliferation and apoptosis. This grant aimed to look at the molecular level to elucidate the mechanisms through which these polyphenols act. Through year two of the project, we have shown that genistein and resveratrol can regulate important molecules in both the growth receptor pathways and the sex steroid receptor pathways at 50 days postpartum. We have now begun to look at the importance of the estrogen receptors in the mechanisms of genistein and resveratrol. Estradiol benzoate was used as a positive estrogenic control and resulted in a significant increase of uterine to body weight ratio. The growth effects may involve the Akt and ERK pathways. Resveratrol, in the ovariectomized model, was able to affect both sex steroid pathways, as well as growth pathways such as EGF and Akt pathways. We have determined that a novel mouse model for mammary cancer, one which over-expresses AIB1, a critical sex steroid coactivator in the mammary gland is not a good model for mammary chemoprevention experiments. The length of time involved in tumorigenesis, lack of early lesions, along with a lack of effect of genistein and resveratrol on the protein expression of AIB1 makes this model

ineffective for chemoprevention experiments. There is much more to learn about the mechanisms of these polyphenols so that they may be used in clinical trials and help women to reduce their risk of breast cancer.

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